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Oxygen regulation of *nifA* transcription *in vitro*

(two-component regulatory system/*fixL–fixJ*/nitrogen fixation/*Rhizobium meliloti*)

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ABSTRACT **In *Rhizobium meliloti*, transcription of the key nitrogen-fixation regulatory genes *nifA* and *fixK* is induced in response to microaerobiosis through the action of the FixL and FixJ proteins. These two proteins are sensor and regulator homologues, respectively, of a large family of bacterial two-component systems involved in sensing and responding to environmental changes. A soluble, truncated form of the membrane protein FixL, FixL*, has been shown to be a hemoprotein that phosphorylates and dephosphorylates FixJ in response to oxygen tension. Here we use an *in vitro* transcription system to prove that FixJ is a transcriptional activator of both *nifA* and *fixK* and that phosphorylation of FixJ markedly increases its activity. Phosphorylation was achieved either by preincubating FixJ with FixL* and ATP or by exposing FixJ to the inorganic phospho donor ammonium hydrogen phosphoramidate. Both FixJ and FixJ-phosphate formed heparin-resistant complexes under the assay conditions used. Lastly, we were able to show that anaerobiosis, in the presence of FixL* and ATP, greatly stimulates FixJ activity at the *nifA* promoter with either *Escherichia coli* or *R. meliloti* RNA polymerase. This use of atmospheric oxygen to control *nifA* transcription *in vitro* represents a reconstitution of a bacterial two-component signal transduction system in its entirety, from effector to ultimate target, by the use of purified components.**

Many nitrogen-fixing organisms, including such distantly related bacteria as *Rhodobacter, Azotobacter, Klebsiella*, and several members of the family *Rhizobiaceae*, contain a conserved regulatory gene designated *nifA* (1–3). The product of this gene, NifA, positively activates the transcription of most nitrogen-fixation genes, including those that encode the subunits of nitrogenase, the enzyme catalyzing the reduction of atmospheric dinitrogen to ammonium (3). Different strategies are employed by various organisms for regulating *nifA* transcription and thus controlling nitrogen fixation. In *Klebsiella pneumoniae*, an enteric bacterium capable of fixing nitrogen under free-living conditions, transcription of *nifA* is induced by the two-component regulatory system NtrB/NtrC when alternative nitrogen sources are in short supply (3). In the soil bacterium *Rhizobium meliloti*, which fixes nitrogen symbiotically within the root nodules of alfalfa (*Medicago sativa*), considerable evidence supports the idea that transcription of both *nifA* and another nitrogen-fixation regulatory gene, *fixK*, is induced in *R. meliloti* by the low free oxygen concentration existing within the root nodule (4–9). In *R. meliloti* cultures grown *ex planta*, microaerobiosis has been shown to strongly induce *nifA* and *fixK* expression (5, 6).

Both in nodules and in free-living cultures, induction of *nifA* and *fixK* is dependent upon the *fixL* and *fixJ* genes, which encode the sensor and regulator, respectively, of a two-component regulatory system (10). FixL and FixJ are members of a large family of sensor/regulator pairs that allow bacteria to respond to various environmental stimuli such as medium osmolarity, plant signals, and nutrient availability. The sensor class is characterized by a conserved C-terminal histidine kinase domain, while the regulator class is characterized by a conserved N-terminal domain that can be phosphorylated at an aspartate residue by its cognate sensor. These regulators are often transcriptional activators, with the phosphorylated form being much more active for transcription (11). Homologues of *fixL* and *fixJ* have been identified in *Bradyrhizobium japonicum*, the endosymbiont of soybean [*Glycine max* (12)], and in *Azorhizobium caulinodans*, the endosymbiont of *Sesbania rostrata* (13).

FixL is a membrane-bound protein (14). A purified soluble derivative of FixL (FixL*) has been shown to be an oxygen-binding hemoprotein capable of autophosphorylation and phospho transfer to FixJ (7) and to possess a FixJ-phosphate phosphatase activity (15). The level of FixJ-phosphate is controlled by the heme-containing FixL* in response to oxygen tension (8). This is achieved through coordinate regulation of the autophosphorylation and phosphatase activities of FixL* (15).

On the basis of N-terminal homology to other two-component regulatory systems, it has been proposed that FixJ is a transcriptional activator of *nifA* and *fixK* expression and that FixJ-phosphate is the active form (10). The observations that low oxygen concentration both increases FixJ-phosphate levels *in vitro* and induces *nifA* and *fixK* expression *in vivo* support this proposal (5, 6, 8). Also, a screen for *fixJ*-defective mutants resulted in the isolation of several point mutants whose gene products were deficient in phosphorylation by FixL* *in vitro* (16).

In this study, we demonstrate that FixJ is a transcriptional activator *in vitro* and that FixL* will substantially increase FixJ activity. In addition, we provide evidence that this increase in activity is due to phosphorylation of FixJ. Finally, we show that reduced oxygen availability substantially enhances the ability of FixL* to stimulate transcription of *nifA* in combination with RNA polymerase from either *R. meliloti* or from the heterologous bacterium *Escherichia coli*.

MATERIALS AND METHODS

Plasmid Constructions (Fig. 1). The transcription templates pPA100 (*nifA*) and pPA200 (*fixK*) are derivatives of pTE103-1. To construct pTE103-1, the pUC derivative pTE103 (17) was first cleaved with *Hin*dIII, the 5′ overhangs were filled in with the Klenow fragment of DNA polymerase I and dNTPs, and ligation was carried out with T4 DNA ligase. After treatment of this intermediate vector with *Eco*RI, the 5′ overhangs were filled in and a *Hin*dIII linker (5′-CAAGCTTG-3′; New England Biolabs) was inserted. The *Hin*dIII–*Bam*HI fragment of pCHK56 (9), spanning *nifA* from bp −108 to bp +193 relative to the transcription start site (9), was transferred to pTE103-1 to yield pPA100, which specifies a hybrid *nifA* transcript with a predicted length of

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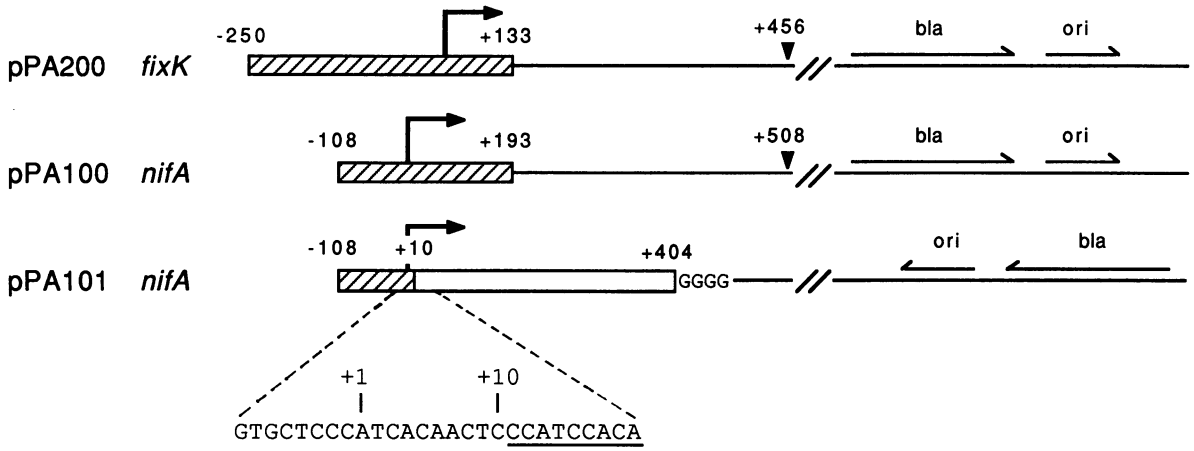

FIG. 1. Relevant features of transcription templates. All coordinates are relative to the transcription start site (+1), depicted here by an arrow indicating the direction of transcription. Predicted transcript length in nucleotides is indicated near the terminator for each template. Hatched bars indicate *Rhizobium* DNA. Filled triangles indicate the position of the Rho-independent transcription terminator from phage T7. Open bar represents a DNA tract with no G residues on the nontranscribed strand. The structure of the *nifA* promoter surrounding the transcription start site in pPA101 is shown. The G at position +4 was changed to an A; underline denotes vector- or construction-related sequences. The translation initiation codon (ATG) for *nifA* and *fixK* is located at +54 and +22, respectively. bla, β-Lactamase gene conferring resistance to penicillin; ori, pUC origin of replication. Each template used in the transcription assay was in the form of supercoiled DNA.

508 bases. Similarly, the *Hin*dIII–*Bam*HI fragment from pPAK2 (18), containing *fixK* sequence from −250 to +133 (6), was inserted in pTE103-1 to generate pPA200, which specifies a *fixK* transcript with a predicted length of 456 bases.

The transcription template pPA101 is a derivative of pμ(−47)-(G−) (19). The *Hin*dIII–*Bam*HI fragment of pCHK56 was transferred to pBluescript SK(−) (Stratagene), whereupon it was mutagenized with the oligodeoxynucleotide 5′-GCACTAACTCCCCCCCGGGAGTTGTGATGG-GAGCACC-3′ according to the method of Kunkel *et al.* (20) to yield a *nifA* promoter with a G → A transition at +4 and a *Sma* I recognition site at +10. The *Kpn* I–*Sma* I fragment from this construct was inserted between the *Kpn* I and *Eco*RV sites of pμ(−47)-(G−) to produce pPA101. This template specifies a G-less transcript with a predicted length of 404 bases. Supercoiled DNA templates were prepared as described (21).

Purification of FixJ and FixL*. FixJ was purified as described (16) except that the ammonium sulfate precipitation was replaced by concentration solely with Centriprep-10 microconcentrators (Amicon). FixL*, purified as described (8), was a gift from M. Weinstein and A. Lois (University of California at San Diego, LaJolla, CA). Both the FixJ and FixL* preparations were >95% pure and were stored at −70°C.

Purification of *R. meliloti* RNA Polymerase. *R. meliloti* 102F34 cells (3.3 g) that had been grown to late stationary phase in TY medium (4.5 mM $CaCl_2$ with 6 g of tryptone and 1 g of yeast extract per liter) supplemented with 0.1% glutamate were sonicated for 15 min at 2–4°C in 20 ml of 100 mM Tris·HCl, pH 7.9/10 mM $MgCl_2$/5 mM EDTA/100 mM KCl/5% (vol/vol) glycerol containing phenylmethylsulfonyl fluoride at 50 μg/ml. The supernatant was diluted to 60 ml with sonication buffer and centrifuged at 64,000 × *g* for 30 min. Polymin P (BASF) was added to the supernatant to 0.25% and the sample was incubated on ice for 15 min. After centrifugation at 12,100 × *g* for 10 min, the pellet was extracted at 0°C for 10 min with 20 ml of 0.6 M NaCl. After additional centrifugation at 12,100 × *g* for 10 min, ammonium sulfate was added to the supernatant to 80% saturation and the mixture was centrifuged at 27,000 × *g* for 10 min. The pellet was dissolved in 4 ml of TGED (10 mM Tris·HCl, pH 7.9/0.1 mM EDTA/0.1 mM dithiothreitol/5% glycerol), diluted to an ionic strength equivalent to 0.25 M NaCl, and loaded onto a heparin-Sepharose (Pharmacia) column equilibrated with TGED/0.25 M NaCl. After extensive washing, the polymerase was batch-eluted with TGED/0.6 M NaCl. Peak absorbing fractions were pooled (11 ml) and concentrated in a Centriprep-30 concentrator (Amicon) to 1.75 ml. The concentrate was chromatographed on a Sephacryl S-200 (Pharmacia) column equilibrated with TGED/0.5 M NaCl. Peak absorbing fractions were pooled and dialyzed against TGED containing 50% glycerol instead of 5% glycerol. Samples were aliquoted and stored at −70°C. Purity was estimated at 95%.

***In Vitro* Transcription Assays.** Phosphorylation reactions were carried out in a base reaction mixture containing 50 mM Tris·HCl (pH 8.0), 0.8 mM $MgCl_2$, 50 mM KCl, 0.8 mM ATP, 1.2 mM $CaCl_2$, 1 mM dithiothreitol, 0.05% bovine serum albumin (Boehringer Mannheim), and 27 nM template. Purified FixL* or FixJ, stored in L buffer (125 mM NaCl/20 mM Tris·HCl, pH 7.8/5% glycerol), was added in the desired amount to obtain a 15-μl reaction volume. After sufficient incubation time, RNA polymerase was added to 145 nM for *E. coli* (100% σ-saturated, Epicentre Technologies, Madison, WI) or 330 nM for *R. meliloti* (it is assumed that *R. meliloti* RNA polymerase has the same molecular weight as *E. coli* RNA polymerase). Polyethylene glycol (average M_r 3000) to 7.5% or additional $MgCl_2$ to 10 mM was added where specified (see figure legends) and the volume was increased to 20 μl. After 10 min at 23°C, a single round of transcription was initiated by adding the following components to a final volume of 25 μl: for pPA100 and pPA200, 720 μM ATP, 400 μM UTP, 400 μM GTP, 100 μM CTP, [α^{32}P]CTP at 2500 cpm/pmol of total CTP, 400 μg of heparin per ml, and 10 mM $MgCl_2$ (if not previously added); for pPA101 the reactions were performed identically except that FPLC-purified UTP and CTP (Promega) were added to 400 μM and 100 μM, respectively. FPLC-purified ATP was already present in the reaction mix (see above for base reaction mixture). After 15 min, the transcription reactions were terminated by the addition of 155 μl of 10 mM Tris·HCl, pH 8.0/3 mM EDTA/0.2% SDS. After phenol extraction and ethanol precipitation in the presence of 10 μg of glycogen (Boehringer Mannheim), the samples were electrophoresed in an 8 M urea/6% polyacrylamide gel and visualized by autoradiography. For quantitation, autoradiograms were scanned with an LKB Ultroscan XL laser densitometer and the data were processed by using software from AMBIS Systems (San Diego).

RESULTS

FixJ Is a Transcriptional Activator. To determine whether FixJ is capable of initiating transcription at the *nifA* and *fixK* promoters of *R. meliloti*, the protein was purified from *E. coli* and tested in an *in vitro* transcription assay (Fig. 2) using the supercoiled form of plasmid constructs prepared for this purpose (Fig. 1). Each plasmid contains a Rho-independent transcription terminator located several hundred nucleotides downstream from the previously characterized transcriptional start sites of *nifA* and *fixK*. *E. coli* RNA polymerase holoenzyme was chosen for these experiments because the *nifA* and *fixK* promoters display *fixJ*-dependent induction in this heterologous host (22). Also, there is evidence that expression of *fixK* in *E. coli* requires σ^{70}, the major σ factor (23). In addition to the *nifA* and *fixK* promoters, a *nifA* promoter mutant was also examined. In this mutant, the sequence AAT at nucleotide positions −53 to −51 was changed to GCG, rendering the promoter defective during both microaerobic growth and symbiosis (18). Addition of FixJ to reaction mixtures containing the wild-type *nifA* and *fixK* promoters (Fig. 2, lanes 4 and 6) caused the appearance of specific RNAs. No detectable RNA was visible at the appropriate positions when FixJ was omitted from the reactions (lanes 3 and 5). Furthermore, there were no apparent transcripts of the expected size from the mutant *nifA* promoter, in either the presence or the absence of FixJ (lanes 1 and 2). We conclude that FixJ is a transcriptional activator of *R. meliloti nifA* and *fixK* and that FixJ and RNA polymerase are necessary and sufficient protein components for this activation. It is not surprising that purified, unphosphorylated FixJ can serve as a transcriptional activator, since a basal level of activity has been observed in similar situations for other response regulators (24–26). Additional experiments using a lower concentration of FixJ substantiated that FixJ-phosphate was much more active than unphosphorylated FixJ (see below). It is noteworthy that the concomitant addition of heparin and NTPs does not decrease specific transcription, suggesting that stable, presumably open complexes are formed. Polyethylene glycol, although not essential, significantly increased the signals obtained (data not shown), most likely through macromolecular crowding (27).

Effect of FixL* and Phosphoramidate on Transcription. Since FixL* will phosphorylate FixJ *in vitro* (7), the effect on transcription of preincubation with this sensor protein was examined (Fig. 3). Our approach was first to lower the FixJ concentration by a factor of 5 from that used for Fig. 2, to a level that substantially reduced the level of transcription of *nifA* and eliminated transcription of *fixK* (Fig. 3, lanes 3). The effect of the addition of FixL* was then observed. FixL* alone was unable to activate transcription from the *nifA* or *fixK* promoter (lanes 2). However, when the two regulatory proteins were combined and preincubated under conditions known to produce FixJ-phosphate, appreciable levels of transcription were observed from both promoters (lanes 4). This increase in transcription was completely dependent on preincubation of FixL* and FixJ in the presence of ATP. When ATP was omitted from the preincubation step, no stimulation of transcription was observed (lanes 5), indicating

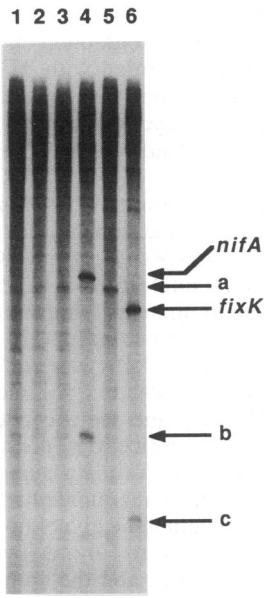

FIG. 2. *In vitro* transcription with purified FixJ. All templates were present at 27 nM. Lanes 1 and 2 show transcription from a template identical to pPA100 except for a mutation in the *nifA* promoter (see text); lanes 3 and 4 show transcription from the *nifA* promoter of pPA100; lanes 5 and 6 show transcription from the *fixK* promoter of pPA200. The reaction mixtures of lanes 2, 4, and 6 contained FixJ at a final concentration of 18.75 μM added in 5 μl of L buffer (125 mM NaCl/20 mM Tris·HCl, pH 7.8/5% glycerol), whereas 5 μl of L buffer alone was added to the reaction mixtures of lanes 1, 3, and 5. Mixtures were incubated for 15 min at 23°C prior to the addition of *E. coli* RNA polymerase, polyethylene glycol, and additional $MgCl_2$ as described in *Materials and Methods*. A band (a) is present in lane 5 in the absence of FixJ activation (see *Discussion*). In addition, a *nifA*-specific band (b) and a *fixK*-specific band (c) are present under inducing conditions and may be the result of premature termination in the vector sequence. In a comparison with labeled size markers, the approximate length of the *nifA* transcript is 497 bases, close to the predicted length of 508 bases, and the approximate length of the *fixK* transcript is 446 bases, close to the predicted length of 456 bases (data not shown).

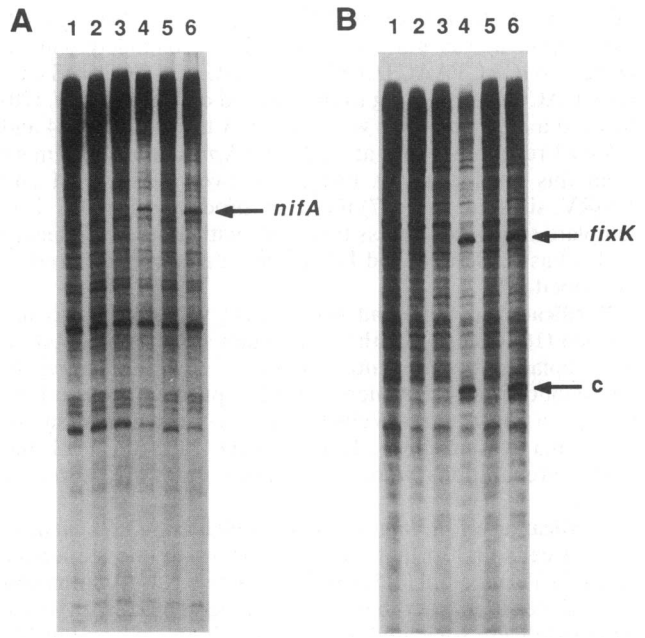

FIG. 3. Stimulation of *nifA* and *fixK* transcription by FixL* and phosphoramidate *in vitro*. Transcription products from pPA100 (*nifA* promoter) (*A*) and pPA200 (*fixK* promoter) (*B*) are shown. For both *A* and *B*: lanes 1, no FixL* or FixJ; lanes 2, FixL* at 1.1 μM; lanes 3, FixJ at 3.75 μM; lanes 4, FixL* at 1.1 μM and FixJ at 3.75 μM combined; lanes 5, identical to lanes 4 except no ATP was present prior to the transcription reaction; lanes 6, FixJ at 3.75 μM with 10 mM phosphoramidate and no ATP present prior to transcription. Reaction mixtures were incubated for 15 min at 23°C prior to the addition of *E. coli* RNA polymerase and additional $MgCl_2$ as described in *Materials and Methods*. All reaction mixtures contained 3 μl of L buffer. Ammonium hydrogen phosphoramidate was synthesized by the method of Sheridan *et al.* (28). A *fixK*-specific band (c), is present under inducing conditions and may be the result of premature termination in the vector sequence.

that the stimulatory effect of FixL* under these conditions is the result of FixJ phosphorylation.

Additional evidence supporting a key role for phosphorylation in this system was obtained by examining the effect of a low molecular weight inorganic phospho donor, ammonium hydrogen phosphoramidate ($NH_4HPO_3NH_2$; ref. 28). This compound phosphorylates the FixJ homologues CheY and CheB of *E. coli* at the appropriate aspartate residue for each protein (29). Phosphoramidate strongly induced FixJ-dependent transcription from both the *nifA* and *fixK* promoters in the absence of FixL* and ATP (Fig. 3, lanes 6). The data presented in Fig. 3 indicate that the ability of FixJ to function as a transcriptional activator is greatly enhanced by phosphorylation of the protein.

Anaerobic Induction of *nifA* Transcription. To examine the levels of transcription from the *nifA* promoter under various conditions with a minimum of background transcripts, the plasmid pPA101 was constructed. In this template, the *nifA* promoter has been fused to a DNA tract lacking G residues in the resulting nontranscribed strand (Fig. 1). With a G → A transition at +4, this allows transcription to proceed using only ATP, CTP, and UTP as substrates, thereby eliminating background transcription from other plasmid sequences. The first G residue encountered by the polymerase provides for efficient termination. Although the initial characterization of transcription was carried out with *E. coli* RNA polymerase (Figs. 2 and 3), RNA polymerase holoenzyme from *R. meliloti* was subsequently purified so that signal transduction could also be studied in a system that reflects the *in vivo* situation as faithfully as possible. This preparation showed activity comparable to that of *E. coli* polymerase when tested with a constitutive *E. coli* σ^{70} promoter on pTE103 in an *in vitro* transcription assay (data not shown).

The effect of anaerobiosis on *in vitro* transcription is shown in Fig. 4. A modest amount of transcription was observed at the FixJ concentration used in these experiments and this amount was not affected by anaerobiosis (Fig. 4, lanes 1 vs. lanes 2). Aerobic preincubation with phosphoramidate markedly stimulated transcription when *R. meliloti* RNA polymerase was used (Fig. 4*B*, lane 0). Addition of FixL* under aerobic conditions increased the signal slightly when *E. coli* RNA polymerase was used (Fig. 4*A*, lanes 1 and 2 vs. lane 3), but not when *R. meliloti* polymerase was used (Fig. 4*B*, lanes 1 and 2 vs. lane 3). Under anaerobic conditions, the addition of FixL* produced a marked increase in transcription with both polymerases (Fig. 4*A*, lane 3 vs. lane 4; Fig. 4*B*, lane 3 vs. lane 4). This demonstrates that transduction of the low-oxygen signal to the transcription of *nifA* has been achieved *in vitro* with only three proteins: FixL*, FixJ, and RNA polymerase.

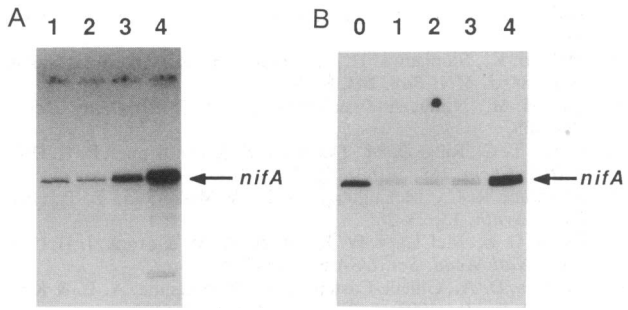

FIG. 4. Anaerobic induction of *nifA* transcription *in vitro* in the presence of *E. coli* RNA polymerase (*A*) or *R. meliloti* RNA polymerase (*B*). The template used was pPA101. All reaction mixtures contained 1.5 μM FixJ and 5 μL of L buffer. Lanes 1, aerobic; lanes 2, anaerobic; lanes 3, FixL* at 0.33 μM, aerobic; lanes 4, FixL* at 0.33 μM, anaerobic; lane 0, aerobic, phosphoramidate at 10 mM and $MgCl_2$ at 10 mM at time 0. Phosphoramidate stimulation is also observed when *E. coli* RNA polymerase is used (Fig. 3). Anaerobic reactions were set up with degassed reagents and were kept under nitrogen gas for 7.5 min prior to the addition of RNA polymerase and polyethylene glycol, at which point the samples were exposed to air. All reactions were carried out at 15°C prior to the addition of RNA polymerase and polyethylene glycol, at which point they were shifted to 23°C. Addition of ATP was time 0. In a comparison with labeled size markers, the approximate length of the *nifA* transcript from pPA101 is 416 bases, close to the predicted length of 404 bases (data not shown).

DISCUSSION

The data presented here show that FixJ is a transcriptional activator of both *nifA* and *fixK*. FixJ-dependent transcription was substantially increased in an ATP-dependent manner by preincubation with a soluble derivative of FixL, FixL*, suggesting that such stimulation is due to FixJ phosphorylation. Consistent with this, FixJ activity was similarly stimulated by ammonium hydrogen phosphoramidate, a low molecular weight inorganic compound capable of phosphorylating the two-component response regulators CheY and CheB (29). Together, these results indicate that the primary role of FixL* in transcription is likely to be in controlling the level of FixJ-phosphate. Reduced oxygen availability has been shown to be a sufficient signal for FixL* to increase its rate of FixJ phosphorylation (8) and to decrease its rate of FixJ-phosphate dephosphorylation (15). Data presented here show that anaerobiosis significantly increases the level of FixJ-dependent transcription from the *nifA* promoter in the presence of FixL* and that this increase is observed with both *E. coli* and *R. meliloti* RNA polymerases. Finally, it should be noted that the truncated form of FixL used in these experiments, FixL*, has been found to show oxygen regulation *in vivo* in the heterologous host, *E. coli* (P.G.A., unpublished results).

The observation that unphosphorylated FixJ can activate transcription in the absence of effectors is consistent with the behavior of other response regulators that have been examined *in vitro*, such as NtrC [nitrogen regulation (24)], OmpR [osmolarity response (26)], and PhoB [phosphate regulation (25)]. Although FixJ can activate transcription without phosphorylation, this may not reflect a significant property of the protein *in vivo* but, rather, may be a function of the concentration or conditions used here.

Phosphoramidate activation of transcription strongly implicates the pivotal role of phosphorylation in the transcription reaction and supports the view that low molecular weight phospho donors can generally serve to phosphorylate two-component response regulators. It also suggests that FixJ can participate in the catalysis of its own phosphorylation, as has been shown for NtrC (30).

Additional bands were observed in transcription reactions with pPA100 and pPA200 (Fig. 2 and Fig. 3*B*; bands a–c). Minor band a is infrequently observed and is not FixJ-specific (Fig. 2, lane 5 vs. lane 6). Minor band b is FixJ-specific but is not seen when the *nifA* mutant promoter is used (Fig. 2, lane 2). It probably arises from premature termination of the *nifA* transcript. We presume that band c, which is also FixJ-specific, is similarly derived from the *fixK* transcript.

Anaerobiosis dramatically stimulates *nifA* transcription when FixJ is preincubated with FixL* and ATP, suggesting that the entire signal transduction pathway has been successfully reconstituted. It is of interest that closely spaced multiple bands were observed in the transcription reactions shown in Fig. 4. Other transcription reactions with *E. coli* RNA polymerase, in which the preincubation was performed at a Mg^{2+} concentration (8–10 mM) higher than that used in lanes 1–4 (0.8 mM), showed only a single band corresponding to the predominant lower molecular weight transcript in Fig. 4 (data not shown). At a higher Mg^{2+} concentration in the

presence of phosphoramidate, a single *nifA* transcript of this size was also observed in this experiment (Fig. 4*B*, lane 0). It is therefore likely that the additional bands resulted from the lower Mg^{2+} concentration, which was necessary to observe significant activity of *R. meliloti* RNA polymerase with FixL*. At high Mg^{2+} concentrations no transcription was detected from reactions otherwise identical to those shown in lanes 1–4 (data not shown). It is unclear why a lower Mg^{2+} concentration in the preincubation is necessary for *R. meliloti* RNA polymerase activity when FixL* is the phospho donor. Additional work will be necessary to clarify this issue.

Up to a 13-fold increase upon anaerobiosis was observed with *E. coli* RNA polymerase. This probably underrepresents the maximal induction capability of the system for several reasons. First, the transmembrane helices have been found to be important for maximal microaerobic induction *in vivo*, implicating the importance of a membrane environment for FixL function (14). Further, folding of the truncated derivative may only approximate that of the full-length protein, thereby possibly compromising FixL* activity. Additional factors might also be necessary for optimal activity. Lastly, single-round transcription assays were used in these experiments, eliminating an important source of signal amplification.

The work presented here is consistent with kinase-dependent transcription observed *in vitro* in the NtrB/NtrC (31) and EnvZ/OmpR (26) two-component systems. Studies with EnvZ/OmpR showed that osmolarity changes can cause changes in OmpR-phosphate levels in a cell-free system containing a cytoplasmic membrane fraction, but these changes were not observed in a system containing highly purified components (32). The components necessary for this response are therefore not clearly defined.

Reconstitution of the *E. coli* chemotaxis system was achieved using phospholipid vesicles and the purified components Tar, CheA, CheW, CheZ, CheY, and CheB. Changes in CheY and CheB (regulator-class proteins) phosphorylation were demonstrated in response to the effector aspartate (33). The latter case is analogous to the demonstration that anaerobiosis will increase FixJ-phosphate levels *in vitro* in a system of pure components, although in the case of FixJ, only one additional protein, FixL*, is sufficient for this effect (8).

EnvZ possesses an OmpR-phosphate phosphatase activity (34), while NtrB displays an NtrC-phosphate phosphatase activity that is potentiated by GlnB. This NtrC-phosphate phosphatase activity decreases NtrB-dependent transcription *in vitro* (35). FixL* has also recently been shown to possess phosphatase activity that is regulated in response to oxygen (15), suggesting that the transcriptional stimulation observed here is a combination of the coordinately regulated kinase/phosphatase activities of FixL*. The relative contribution of these two activities is unknown and is an important question for further study. It is noteworthy that this report demonstrates signal transduction from a defined stimulus to an effect, in this case transcriptional activation, by using purified components of the native host.

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